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Suppression of NF-κB activation by curcumin leads to inhibition of expression of cyclo-oxygenase-2 and matrix metalloproteinase-9 in human articular chondrocytes: Implications for the treatment of osteoarthritis

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ABSTRACT

Pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) play a key role in the pathogenesis of osteoarthritis (OA). Anti-inflammatory agents capable of suppressing the production and catabolic actions of these cytokines may have therapeutic potential in the treatment of OA and a range of other osteoarticular disorders. The purpose of this study was to examine the effects of curcumin (diferuloylmethane), a pharmacologically safe phytochemical agent with potent anti-inflammatory properties on IL-1β and TNF-α signalling pathways in human articular chondrocytes maintained *in vitro*. The effects of curcumin were studied in cultures of human articular chondrocytes treated with IL-1β and TNF-α for up to 72 h. Expression of collagen type II, integrin β1, cyclo-oxygenase-2 (COX-2) and matrix metalloproteinase-9 (MMP-9) was monitored by western blotting. The effects of curcumin on the expression, phosphorylation and nuclear translocation of protein components of the NF-κB system were studied by western blotting and immunofluorescence, respectively. Treatment of chondrocytes with curcumin suppressed IL-1β-induced NF-κB activation via inhibition of IκBα phosphorylation, IκBα degradation, p65 phosphorylation and p65 nuclear translocation. Curcumin inhibited the IL-1β-induced stimulation of up-stream protein kinase B Akt. These events correlated with down-regulation of NF-κB targets including COX-2 and MMP-9. Similar results were obtained in chondrocytes stimulated with TNF-α. Curcumin also reversed the IL-1β-induced down-regulation of collagen type II and β1-integrin receptor expression. These results indicate that curcumin has nutritional potential as a naturally occurring anti-inflammatory agent for treating OA through suppression of NF-κB mediated IL-1β/TNF-α catabolic signalling pathways in chondrocytes.

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1. Introduction

Pro-inflammatory cytokines such as IL-1β and TNF-α are produced by activated synoviocytes and articular chondrocytes and play a pivotal role in the pathogenesis of osteoarthritis (OA) [1–3]. Once released, these cytokines are potent stimulators for the *de novo* production of catabolic enzymes such as matrix metalloproteinases (MMPs), which are responsible for excessive cartilage matrix degradation in OA [3–5]. IL-1β and TNF-α also activate other mediators of inflammation such as cyclo-oxygenase-2 (COX-2) which increase prostaglandin E_2 (PGE_2)-production, joint pain and further synovial inflammation [5]. IL-1β and TNF-α also suppress the expression of cartilage-specific extracellular matrix components including collagen type II and cartilage-specific proteoglycans. These catabolic changes exacerbate the OA associated loss of cartilage matrix [4,6–9]. In recent years anti-TNF-α therapy has been introduced as a new therapeutic approach for intervention in rheumatoid arthritis [10,11]. Although anti-TNF drugs such as etanercept and infliximab show encouraging results in rheumatoid arthritis patients, some individuals fail to respond to treatment. Moreover, adverse side effects of these drugs have recently come to light, the most important being impairment of the patient's immune and surveillance systems [10,11], which will increase susceptibility to opportunistic bacterial and viral infections and to the spontaneous development of tumors. Therefore, it is of considerable clinical interest to identify novel naturally occurring pharmacotherapies for OA and related osteoarticular conditions.

Studies aimed at unravelling the underlying molecular alterations brought about by pro-inflammatory cytokines in chronic inflammatory conditions have revealed that IL-1β and TNF-α both activate a ubiquitous central transcription factor known as NF-κB, which is a key regulator of gene expression [5,12]. In the inactive state, NF-κB is present in the cytoplasm as a heterotrimer complex consisting of two subunits and an additional inhibitory subunit: IκBα. Five different subunits exist: c-Rel, RelA (also known as p65), RelB, p50/p105, p52/p100 which can form homo- or heterodimers in varying combinations. P65/p50 is one of the most prevalent combinations [13]. During the activation process, the inhibitory subunit IκBα is phosphorylated at Ser 32 and Ser 36 residues by IKK kinase (IκBα kinase) and is subsequently degraded. Once released, subunits of activated NF-κB translocate to the nucleus where they bind NF-κB-recognition (κB) sites in the promoter regions of selected target genes, activating their expression [5,14]. Despite the above advances, the functional role of NF-κB in chondrocytes in OA is unclear and remains to be elucidated.

Many of the biological effects of IL-1β and TNF-α on chondrocytes (i.e. up-regulation of MMPs, COX-2 and inducible nitric oxide synthase) are also mediated by NF-κB [5,15–17]. In other cell types the expression of adhesion molecules such as cell adhesion molecule-I (I-CAM), vascular endothelial growth factor (VEGF), urokinase plasminogen activator (uPa), Bcl-2 and pro-inflammatory cytokines have been shown to be regulated by NF-κB [18,19]. The mechanism of down-regulation of chondrocyte-specific matrix synthesis may also involve NF-κB activation [7,20]. NF-κB appears to be a common downstream target of multiple converging catabolic signalling pathways (e.g. those mediated by IL-1β and TNF-α) [21].

Curcumin is a major component of turmeric, a yellow spice derived from dried rhizomes of *Curcuma longa*. Curcumin has been proposed as a naturally occurring chemotherapeutic agent for cancer therapy since it reduces tumor cell survival, tumor expansion, and secondary inflammation via NF-κB inhibition [19,22,23]. Curcumin has already entered clinical trials because of its potent anti-inflammatory, anti-carcinogenic and free radical scavenger properties [23]. Although curcumin is a potent inhibitor of NF-κB, its effects on articular chondrocytes have not been investigated at the cellular or molecular levels. Accordingly, the aim of this study was to exploit an *in vitro* model of human articular chondrocytes to study the mechanism of curcumin action on TNF-α/IL-1β signalling and test the hypothesis that curcumin antagonizes the catabolic effects of pro-inflammatory cytokines by suppressing NF-κB-activation and NF-κB induced gene expression.

2. Materials and methods

2.1. Antibodies

Antibodies to collagen type II (AB746), β1-integrin, CD 29 (MAB1977) were purchased from Chemicon International (Temecula, CA, USA). Secondary antibodies were purchased from Chemicon International and Dianova (Hamburg, Germany). Antibodies to β-actin (A5316) were from Sigma (Munich, Germany). Antibodies raised against MMP-9 (MAB911) were purchased from R&D Systems (Abingdon, UK). Cyclo-oxygenase-2 (160-112) antibody was obtained from Cayman Chemical (Ann Arbor, MI, USA). Antibodies to p65 (IMG-512), phospho-IκBα (IMG-156A), pan-IκBα (IMG-127), pan-Akt and phospho-Akt were obtained from Biocarta (Hamburg, Germany). Antibodies to NFκB p65 (Rel A) and phospho-specific pS529 (100-401-266) were obtained from Rockland laboratories (Biomol, Hamburg, Germany). Peptide aldehydes, a specific proteosome inhibitor N-Ac-Leu-Leu-norleucinal (ALLN) was obtained from Boehringer Mannheim (Mannheim, Germany).

2.2. Growth medium and chemicals

Growth medium (Ham's F-12/Dulbecco's modified Eagle's medium (50/50) containing 10% fetal calf serum (FCS), 25 μg/ml ascorbic acid, 50 IU/ml streptomycin, 50 IU/ml penicillin, 2.5 μg/ml amphotericin B, essential amino acids and L-glutamine) was obtained from Seromed (Munich, Germany). Curcumin, Alginate, O.C.T. compound embedding medium and trypsin/EDTA (EC 3.4.21.4) were purchased from Sigma (Munich, Germany). Epon was obtained from Plano (Marburg, Germany). IL-1β and TNF-α were kindly provided by Strathman Biotech GmbH (Hannover, Germany). Curcumin was prepared as a 5 mM solution in dimethylsulfoxide and then further diluted in cell culture medium.

2.3. Chondrocyte isolation and culture

Primary cultures of human chondrocytes were isolated from articular cartilage as previously described [24]. Cells were

resuspended in growth medium and cultured at a concentration of 2×10^6 cells/ml in the three-dimensional alginate system as recently described [25]. After a few days of culture, the chondrocytes migrated from alginate and adhered on Petri dishes forming a monolayer [9,26]. These cells (passage 2) were cultured at 0.1×10^6 cells/ml in Petri dishes in monolayer culture and on glass plates for a period of 24 h at 37 °C with 5% CO_2.

2.4. Experimental design

To obtain specific ligation of pro-inflammatory cytokines with their respective receptors on chondrocytes in the absence of any other stimulation that might be caused by other cytokines or growth factors present in bovine serum, human articular chondrocytes were serum starved and exposed to 10 ng/ml IL-1β or TNF-α alone for 24 h before being co-treated with 50 μM curcumin and 10 ng/ml IL-1β for 0, 12, 24, 36 and 48 h. The experiments described in the present study were specifically designed to mimic the cellular events that occur in the clinical condition of OA.

For investigation of NF-κB translocation and IκBα phosphorylation, chondrocyte cultures were treated either with 10 ng/ml IL-1β or co-treated with 10 ng/ml IL-1β and 50 μM curcumin for 0, 10, 15, 30 and 60 min and nuclear and cytoplasmic extracts were prepared. These experiments were performed in triplicate and the results are provided as mean values from three independent experiments.

2.5. Immunofluorescence microscopy

Cells were seeded on glass plates and incubated for 24 h. The cells were then rinsed three times and pre-incubated for 1 h with serum-starved medium before being stimulated with 10 ng/ml IL-1β or 50 μM curcumin alone or being co-treated with 10 ng/ml IL-1β and 50 μM/ml curcumin for 30 min in serum-starved (0.5% FCS) medium. Glass plates with chondrocyte monolayers were rinsed three-times in Hanks solution before methanol fixation for 10 min at ambient temperature (AT), and rinsing with PBS. Cell and nuclear membranes of chondrocytes were permeabilized by treatment with 0.1% Triton X-100 for 1 min on ice. Cells were overlaid with protease-free bovine serum albumin (BSA) for 10 min at AT, rinsed with PBS and incubated with primary antibodies (p65, phospho-p65, 1:30 in PBS) in a humid chamber overnight at 4 °C. They were gently washed several times with PBS before incubation with secondary antibody (goat–anti-rabbit immunoglobulin conjugated with FITC [GAR–FITC], diluted 1:50 in PBS) for 1 h at AT. Cells were finally washed three times with PBS, covered with fluoromount mountant, and examined under a light microscope (Axiophot 100, Zeiss, Germany).

2.6. Isolation of chondrocyte nuclei

Chondrocytes were trypsinized and washed twice in 1 ml ice cold PBS. The supernatant was carefully removed. Cell pellets were re-suspended in 400 μl hypotonic lysis buffer containing protease inhibitors and incubated on ice for 15 min. 12.5 μl of 10% NP-40 was added and the cell suspension was vigorously mixed for 15 s. The extracts were centrifuged for 1.5 min. The supernatants (cytoplasmic extracts) were frozen at −70 °C. 25 microliters of ice cold nuclear extraction buffer was added to the pellets and incubated for 30 min with intermittent mixing. Extracts were centrifuged and the supernatant (nuclear extracts) transferred to pre-chilled tubes for storage at −70 °C.

2.7. Western blot analysis

Chondrocyte monolayers were washed three times with Hank's solution and whole cell proteins were extracted by incubation with lysis buffer (50 mM Tris–HCl, pH 7.2, 150 mM NaCl, l% (v/v) Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium pyrophosphate, 100 mM sodium fluoride, 0.01% (v/v) aprotinin, 4 μg/ml pepstatin A, 10 μg/ml leupeptin, 1 mM PMSF) on ice for 30 min, and cell debris was removed by centrifugation. Supernatants were stored at −70 °C until use. Total protein concentration of whole cell, nuclear and cytoplasmic extracts was determined according to the bicinchoninic acid system (Uptima, Interchim, Montlucon, France) using BSA as a standard. After adjusting the equal amounts (50 μg of protein per lane) of total proteins they were separated by SDS-PAGE (5, 7.5% gels) under reducing conditions. The separated proteins were transferred onto nitrocellulose membranes. Membranes were pre-incubated in blocking buffer (5% (w/v) skimmed milk powder in PBS/0.1% Tween 20) for 30 min, and incubated with primary antibodies (1 h, AT). Membranes were washed three times with blocking buffer, and incubated with alkaline phosphatase conjugated secondary antibodies for 30 min. They were finally washed three times in 0.1 M Tris pH 9.5 containing 0.05 M $MgCl_2$ and 0.1 M NaCl. Nitro blue tetrazolium and 5-bromo-4-chloro-3-indoylphosphate (*p*-toluidine salt; Pierce, Rockford, IL, USA) were used as substrates to reveal alkaline phosphatase conjugated specific antigen–antibody complexes.

3. Results

3.1. Cell culture

Freshly isolated primary human chondrocytes were introduced into three-dimensional alginate culture to preserve the phenotypic characteristics of the cells and to allow the cells to adapt gently to culture conditions *in vitro*. After a few days in culture, some cells migrated from the alginate beads and adhered to the Petri dishes reaching confluence 3 days later. At this point it is important to highlight the rationale for adopting this approach. We have previously shown that alginate migrated cells are fully differentiated chondrocytes and uniquely possess chondrogenic potential. Our ultrastructural studies have demonstrated that alginate culture may be used as a selective filter to separate motile cells with chondrogenic potential from dying and degenerating cells trapped within the alginate bead [9,26]. Migrated chondrocytes exhibited a typical chondrocytic round shape and proliferated rapidly. For all the experiments described these migrated chondrocytes were passaged only twice, to circumvent dedifferentiation. During IL-1β-stimulation, ALLN- and curcumin-treatment, the cells did not show

any morphological features associated with cytotoxicity effects at the light microscopic and ultrastructural levels.

3.2. *Down-regulation of extracellular matrix and signalling proteins by IL-1β or TNF-α on chondrocytes is prevented by curcumin*

Fig. 1A shows serum-starved human articular chondrocytes were stimulated with 10 ng/ml IL-1β for 24 h before co-treatment with 10 ng/ml IL-1β and 50 μM curcumin for 0, 12, 24, 36, 48 h (Fig. 1A). Chondrocytes, stimulated with IL-1β alone showed down-regulation of synthesis of collagen type II in a time-dependent manner (Fig. 1A, I). Synthesis of β1-integrin (CD 29) also decreased in a time-dependent manner (Fig. 1A, II). Co-treatment of chondrocytes with IL-1β and curcumin resulted in an inhibition of cytokine-induced effects on collagen type II and β1-integrin (CD 29) (Fig. 1A, I and II). The same experiments conducted with 10 ng/ml TNF-α instead of IL-1β produced similar results (data not shown). Synthesis of the house-keeping protein β-actin remained unaffected in chondrocytes exposed to curcumin (Fig. 1A, III). To examine that the cytokine induced alterations in the last 48 h were not due to a general adaptation of the cellular protein synthetic machinery to the catabolic effects of IL-1β *in vitro*, we performed additional time-course experiments exclusively with IL-1β (without curcumin) (Fig. 1B). Additionally, to show the effects of curcumin alone we performed time–course experiments with 50 μM curcumin in the absence of IL-1β for 72 h (Fig. 1C). Chondrocytes, stimulated with IL-1β alone showed down-regulation of synthesis of collagen type II (Fig. 1B, I) and of β1-integrin (CD 29) (Fig. 1B, II) in a time-dependent manner even in the final 48 h. Treatment of chondrocytes with 50 μM curcumin alone resulted in stimulation of collagen type II (Fig. 1C, I) and β1-integrin (CD 29) even in the final 48 h (Fig. 1C, II). Synthesis of the house-keeping protein β-actin remained unaffected (Fig. 1B–C, III).

3.3. *Up-regulation of pro-inflammatory enzymes by IL-1β or TNF-α on chondrocytes is inhibited by curcumin*

Serum-starved human articular chondrocytes were exposed to 10 ng/ml IL-1β alone for 24 h before being co-treated with 50 μM curcumin and 10 ng/ml IL-1β for 0, 12, 24, 36, 48 h and the whole cell extracts were prepared and analysed by western blot analysis for the experiments of COX-2 and MMP9 (Fig. 2A). In response to 10 ng/ml IL-1β chondrocytes showed up-regulation

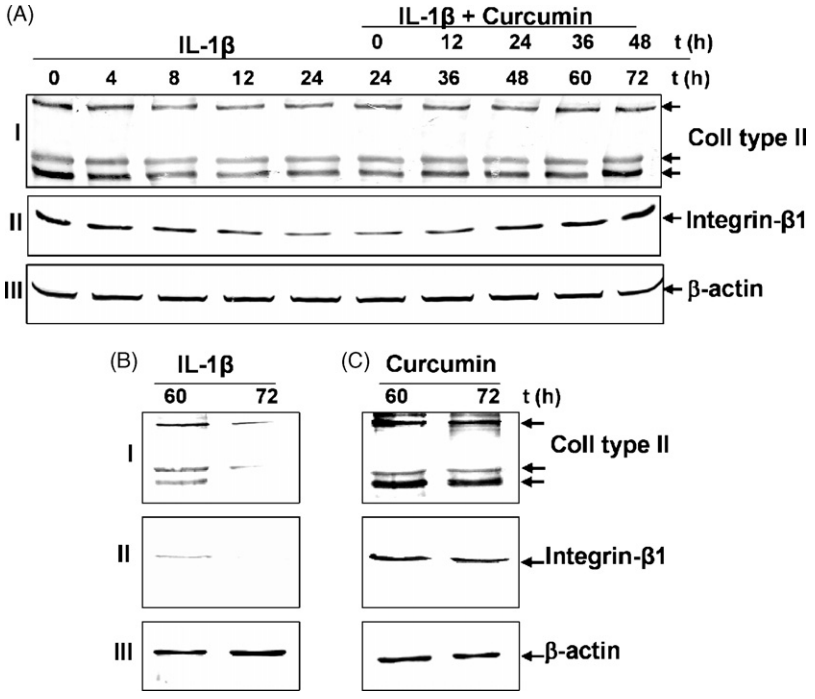

Fig. 1 – Down-regulation of extracellular matrix and signalling proteins by IL-1β in chondrocytes is prevented by curcumin. (A) Serum-starved human articular chondrocytes (0.1×10^6 cells/ml) were exposed to 10 ng/ml IL-1β alone for 24 h before being co-treated with 50 μM curcumin and 10 ng/ml IL-1β for 0, 12, 24, 36, 48 h. Results of western blot analysis revealed down-regulation of collagen type II (I) and β1-integrin (II) in chondrocytes by IL-1β in a time-dependent manner. Co-treatment of chondrocytes pre-stimulated with IL-1β and curcumin relieved the IL-1β-induced inhibition of collagen type II and β1-integrin (I and II). Expression of β-actin was not affected by IL-1β and/or curcumin (III). Serum-starved chondrocytes (0.1×10^6 cells/ml) were exposed to 10 ng/ml IL-1β alone for 72 h (B) or with 50 μM curcumin alone for 72 h (C). Results of western blot analysis revealed a time-dependent down-regulation of collagen type II and β1-integrin in chondrocytes stimulated with IL-1β alone (B, I and II, here we show only the last two time points 60 and 72 h). Treatment of chondrocytes with curcumin restored expression of collagen type II and β1-integrin (C, I and II, again, only the last two time points 60 and 72 h are shown). Expression of β-actin was not affected by IL-1β and/or curcumin (B and C, III). Data shown are representative of three independent experiments.

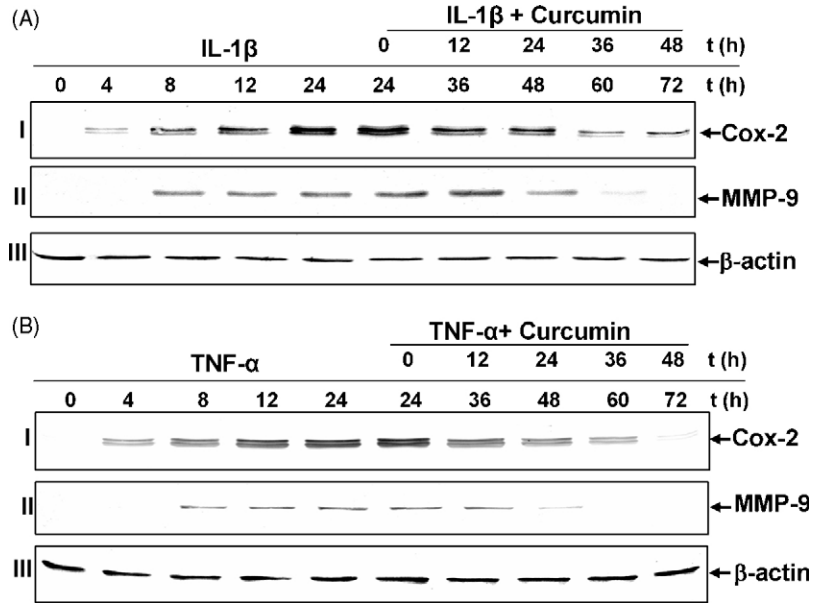

Fig. 2 – Up-regulation of pro-inflammatory enzymes by IL-1β in chondrocytes is inhibited by curcumin. (A) Serum-starved chondrocytes (0.1×10^6 cells/ml) were exposed to 10 ng/ml IL-1β alone for 24 h before being co-treated with 50 μM curcumin and 10 ng/ml IL-1β for 0, 12, 24, 36, 48 h. IL-1β-stimulation led to an increase in synthesis of COX-2 and MMP-9 (I and II). In contrast to this, COX-2- and MMP-9-up-regulation was inhibited by curcumin in chondrocytes pre-stimulated with 10 ng IL-1β for 24 h before being co-treated with 10 ng/ml IL-1β + 50 μM curcumin (A, I and II) as revealed by western blot analysis. Expression of the house-keeping gene β-actin was not affected by treatment with IL-1β and/or curcumin (A, III). (B) (I–III) Up-regulation of pro-inflammatory enzymes by TNF-α on chondrocytes was inhibited by curcumin. Serum-starved chondrocytes (0.1×10^6 cells/ml) were pre-stimulated with 10 ng/ml TNF-α alone for 24 h before being co-treated with 50 μM curcumin and 10 ng/ml TNF-α for 0, 12, 24, 36, 48 h (I and II). TNF-α-treatment led to an increase in synthesis of COX-2 and MMP-9 (B, I and II). COX-2- and MMP-9-up-regulation was inhibited by curcumin in chondrocytes pre-stimulated with 10 ng TNF-α (B, I and II) as revealed by western blot analysis. Expression of the house-keeping gene β-actin was not affected by treatment with TNF-α and/or curcumin (B, III). Data shown are representative of three independent experiments.

of synthesis of COX-2 (Fig. 2A, I) and MMP-9 (Fig. 2A, II) in a time-dependent manner. Co-treatment of IL-1β-pre-stimulated chondrocytes with a combination of IL-1β and curcumin led to a decrease in COX-2 and MMP-9 expression (Fig. 2A, I and II). Synthesis of the house-keeping protein β-actin remained unaffected (Fig. 2A, III). Experiments performed in the same manner using 10 ng/ml TNF-α instead of IL-1β produced comparable results (Fig. 2B, I and II). To ensure that the cytokine induced alterations in the last 48 h were not due to a general adaptation of the cellular protein synthetic machinery to the catabolic effects of IL-1β or TNF-α *in vitro*, we performed additional time–course experiments exclusively with IL-1β or TNF-α (without curcumin) (data not shown).

3.4. *Curcumin blocks phosphorylation and translocation of p65 by IL-1β*

3.4.1. *Effect of curcumin on IL-1β-induced phosphorylation of NF-κB in the cytoplasm*

To test the effect of curcumin on IL-1β activation of NF-κB, serum-starved chondrocytes were treated with 10 ng/ml IL-1β for 0, 10, 15, 30, 60 min alone and other cultures were treated first with 10 ng/ml IL-1β for the same time periods alone and every time course was co-treated with 50 μM curcumin for 4 h. Cytoplasmic extracts were investigated for expression of pan/phospho p65 (Fig. 3A, I and II) by western blot analysis. It is well known, that phosphorylation of p65 is a prerequisite for the NF-κB transcriptional functions and that this phosphorylation is mediated by IKK [28]. As shown in Fig. 3A, IL-1β-induced phosphorylation of the p65 cytoplasmic pool in a time-dependent manner. This phosphorylation could be observed as early as 5 min and increased up to 30 min (Fig. 3A, II). In chondrocytes, which were treated with curcumin, the IL-1β-induced activation of cytoplasmic p65 was blocked completely (Fig. 3A, II).

3.4.2. *Effects of curcumin on IL-1β-induced phosphorylation of p65 in the nucleus*

Translocation of NF-κB to the nucleus is necessary for regulation of gene expression by NF-κB. The translocation of activated NF-κB is preceded by phosphorylation of the p65 subunit of NF-κB [13]. Therefore, to test this, protein extracts of serum-starved chondrocyte nuclear extracts were probed for the pan and phosphorylated p65 NF-κB-subunit after stimulation of chondrocytes with 10 ng/ml IL-1β for 0, 10, 15, 30, 60 min (Fig. 3B, I and II). To show the effect of curcumin on phosphorylation of p65, other cultures were treated first with 10 ng/ml IL-1β alone for the same time periods and then every

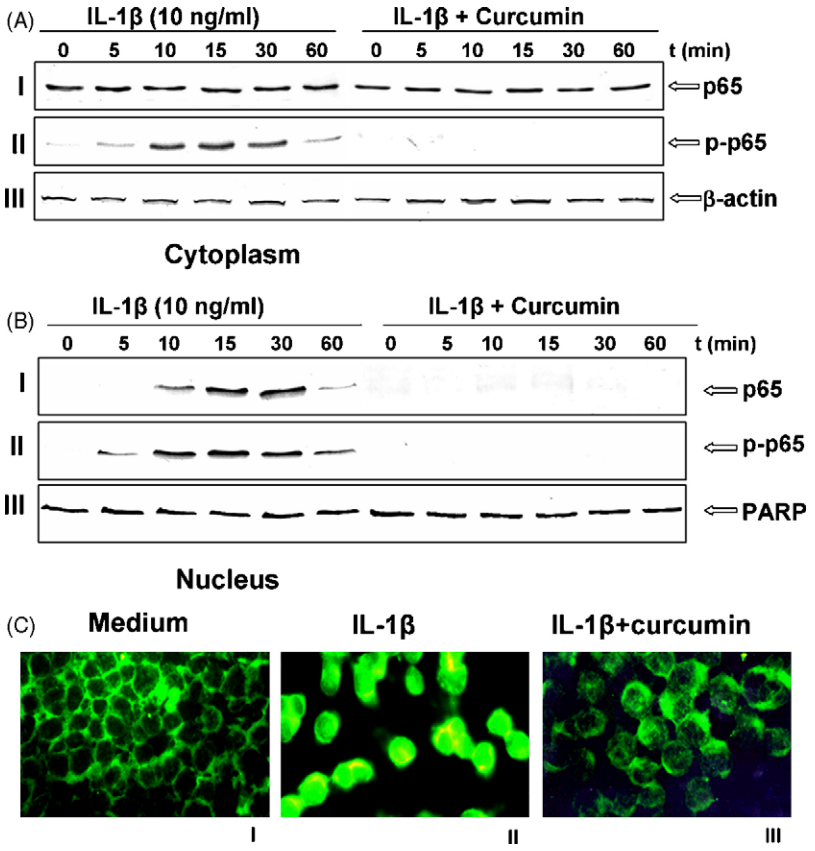

Fig. 3 – Curcumin inhibited IL-1β-induced phosphorylation and translocation of p65 in cytoplasmic and nuclear extracts of chondrocytes. (A) Western blot analysis with IL-1β-treated cytoplasmic extract. Serum-starved chondrocytes (0.1×10^6 cells/ml) were treated with 10 ng/ml IL-1β for 0, 10, 15, 30 and 60 min. Other cultures were initially treated with 10 ng/ml IL-1β for identical time periods and then co-treated with 50 μM curcumin for 4 h. Cytoplasmic extracts were probed for expression of pan/phospho p65 (A, I and II) by western blot analysis using pan antibodies to p65 and phospho-specific antibodies to p65. Pan p65 remained unaffected by IL-1β and co-treatment in cytoplasmic extracts (I). The level of phosphorylation of p65 in the cytoplasmic pool increased in a time-dependent manner. This phosphorylation was observed as early as 5 min after IL-1β treatment and increased up to 30 min (II). The phosphorylation of p65 in the cytoplasmic pool in cultures co-treated with curcumin was completely blocked (II). Synthesis of β-actin remained unaffected in cytoplasmic extracts (III). (B) Western blot analysis with IL-1β-treated nuclear extract. Cells were treated as described in (A). Nuclear extracts were probed for pan/phospho p65 (B, I and II) by western blot analysis using antibodies to p65, phospho-specific p65 and PARP (control). Treatment of chondrocytes with 10 ng/ml IL-1β revealed a clear time-dependent increase in expression of pan and phospho p65 in the nuclear extracts (B, I and II). Co-treatment of chondrocytes with curcumin completely abolished the pan p65 and the IL-1β-dependent activation of phospho p65 in the nucleus (B, I and II). Synthesis of PARP remained unaffected in nuclear extracts (III). (C) Curcumin inhibited IL-1β-induced nuclear translocation of phospho p65 as revealed by immunofluorescence microscopy. Chondrocyte cultures either served as controls (I, not treated) or were treated with curcumin alone for 10 min (II) or co-treated with 10 ng/ml IL-1β + 50 μM curcumin for 1 h (III) before immunolabelling with phospho p65 antibodies and FITC-coupled secondary antibodies. In control cells anti-phospho p65 labelling was restricted to the cytoplasm (I). Cells treated with IL-1β alone revealed nuclear translocation of phospho p65 (II) that was partly inhibited by co-treatment with curcumin (III). (B) I–III 160×. Data shown are representative of three independent experiments.

time course was co-treated with 50 μM curcumin for 4 h (Fig. 3B, I and II). Treatment of chondrocytes with 10 ng/ml IL-1β at different time points visibly revealed an increase in pan p65 subunit and phospho p65 in a time-dependent manner in the nuclear extracts (Fig. 3A, I and II). The co-treatment of chondrocytes with curcumin and IL-1β abolished the pan p65 subunit and the IL-1β-dependent phosphorylation of p65 in a time-dependent manner in the nucleus (Fig. 3B, I and II). These results show quite clearly that curcumin inhibits the IL-1β-induced translocation of p65 to the nucleus. The synthesis of the PARP protein remained unaffected (Fig. 3B, III).

3.4.3. *Curcumin inhibits nuclear-translocation of p65 revealed by IL-1β*

Immunofluorescence microscopy was employed to reveal translocation of phosphorylated p65 subunit of NF-κB from the

chondrocyte cytoplasm to the nucleus in response to NF-κB-activation by IL-1β. Chondrocytes remained either unstimulated (Fig. 3C, I) or were stimulated with curcumin alone (not shown) or with 10 ng/ml IL-1β alone for 10 min (Fig. 3C, II) or were co-treated with for 10 ng/ml IL-1β 10 min and then 50 μM curcumin for 1 h (Fig. 3C, III) before indirect immunolabelling with anti-pan and anti-phospho p65 antibody. FITC-coupled secondary antibodies were used. Pan p65 labelling was restricted to the cytoplasm in control and treated cultures (not shown). Control chondrocytes and chondrocytes treated with curcumin alone (not shown) showed only cytoplasmic labelling of phospho p65 (Fig. 3C, I). IL-1β-stimulated cells revealed clear and intensive cytoplasmic and nuclear staining for phospho p65 (Fig. 3C, II). Co-treatment of chondrocytes with IL-1β and curcumin resulted in inhibition of nuclear transition of activated phospho p65 and decreased cytoplasmic staining for this protein and showed a decrease in activation of NF-κB (Fig. 3C, III). These immunomorphological findings were consistent with the NF-κB inhibition observed by western blotting.

3.5. *Curcumin inhibits IL-1β-dependent IκBα phosphorylation*

Thus far our results demonstrate that curcumin blocks IL-1β-induced activation of NF-κB and its translocation to the chondrocyte nucleus. We proceeded to determine more precisely the upstream kinetics of NF-κB activation by IL-1β in chondrocytes. It is well known that an essential prerequisite for the activation of NF-κB is the phosphorylation and degradation of IκBα, the natural blocker of NF-κB [29]. To test whether inhibition of IL-1β-induced NF-κB activation occurs

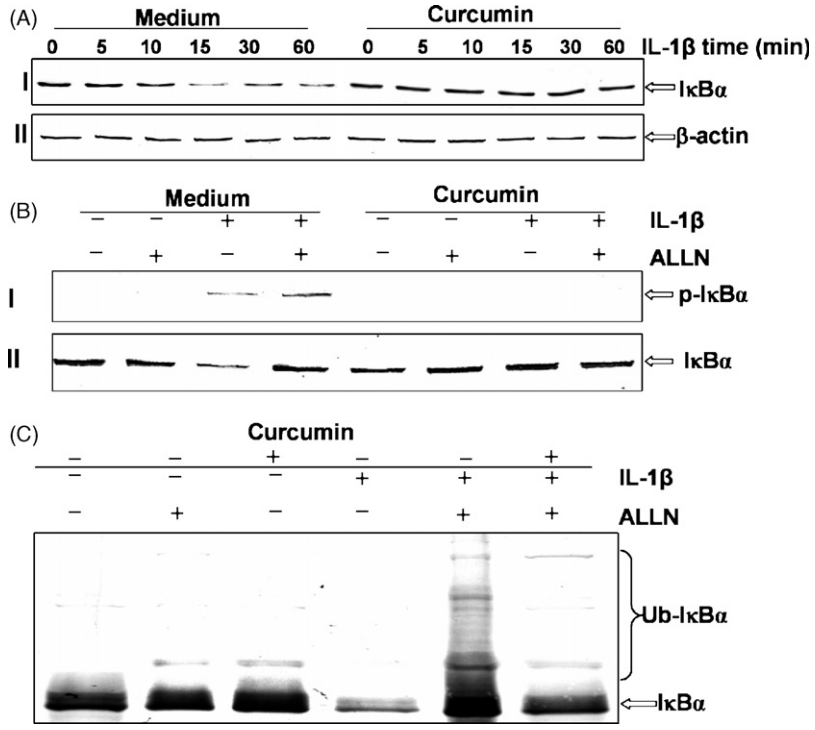

Fig. 4 – Effects of curcumin on the kinetics of IκBα by IL-1β in chondrocytes. (A) Effect of curcumin on IL-1β-induced degradation of IκBα. Serum-starved chondrocytes (0.1×10^6 cells/ml) were treated with 10 ng/ml IL-1β for the indicated times. Other cultures were initially treated with 10 ng/ml IL-1β for identical time periods and then every time course was co-treated with 50 μM curcumin for 4 h. Cytoplasmic extracts were probed for expression of IκBα by western blot analysis using antibodies to IκBα. Expression of β-actin was used as an internal control. IL-1β caused IκBα degradation in cultures as early as 10 min after IL-1β treatment. In co-treated cultures, the degradation of IκBα was not observed (A, I). Synthesis of β-actin remained unaffected (II). (B) Effect of curcumin on the phosphorylation of IκBα by IL-1β. Serum-starved chondrocytes (0.1×10^6 cells/ml) were pre-treated with 100 μg/ml ALLN for 30 min and co-treated with curcumin (100 μM) for 4 h and stimulated with IL-1β (10 ng/ml) for 1 h. Following fractionation of cytoplasmic extracts, western blot analysis was performed using anti-IκBα and p-IκBα antibodies. Treatment of chondrocytes with 10 ng/ml IL-1β revealed an increase in the phosphorylated IκBα-form in cytoplasmic extracts. In the presence of the inhibitor phosphorylation of IκBα was significantly increased (I). Phosphorylation of IκBα was strongly inhibited in cultures co-treated with curcumin in the presence or absence of the inhibitor (I). (C) Effect of curcumin on IL-1β-induced ubiquitination of IκBα. Serum-starved chondrocytes (0.1×10^6 cells/ml) were pre-treated with 100 μg/ml ALLN for 30 min, co-treated with curcumin (100 μM) for 4 h and stimulated with IL-1β (10 ng/ml) for 1 h. Whole cell extracts were prepared followed by western blot analysis using antibodies to IκBα. Treatment with IL-1β alone induced minor ubiquitination of IκBα, but in chondrocytes co-treated with ALLN and IL-1β, ubiquitination of IκBα was significantly increased. Curcumin inhibited the IL-1β-induced IκBα ubiquitination, in the presence or absence of inhibitor. Data shown are representative of three independent experiments.

through inhibition of IκBα degradation, we treated serum starved chondrocytes with 10 ng IL-1β for the indicated times and other cultures were treated first with 10 ng/ml IL-1β for the same time periods alone and then every time course was co-treated with 50 μM curcumin for 4 h. The activation of IκBα in the cytoplasm of chondrocytes was determined by western blot analysis using anti-IκBα and anti-β-actin (control) antibodies. IL-1β caused IκBα degradation in untreated cultures as early as 10 min. In co-treated cultures the degradation of IκBα was not evident (Fig. 4A, I). Taken together, these results suggest that curcumin blocks the IκBα degradation by IL-1β.

To test whether curcumin is able to block the IL-1β-induced phosphorylation of IκBα serum starved chondrocytes were treated with IL-1β for 1 h and examined by western blot analysis using an antibody which is able to recognize the phosphorylated form of IκBα. It is known that phosphorylation of IκBα leads to its degradation [29], the phosphorylation and degradation of IκBα were inhibited by a specific proteosome inhibitor N-Ac-Leu-Leu-norleucinal (ALLN) [30]. As shown in Fig. 4B, IL-1β was able to phosphorylate IκBα in cells pre-treated with the inhibitor; the phosphorylation of IκBα was significantly higher compared to control cells. Interestingly, curcumin was able to inhibit the phosphorylation of IκBα induced by IL-1β in the presence or absence of the inhibitor.

IκBα phosphorylation by pro-inflammatory cytokines leads to ubiquitination and degradation of IκBα [29]. To test whether curcumin influences IL-1β-induced IκBα-ubiquitination, the serum starved chondrocytes were treated with proteosome inhibitor ALLN to inhibit the IκBα degradation [30]. Chondrocytes treated with IL-1β alone induced a low amount of ubiquitination of IκBα; in contrast when the chondrocytes were co-treated with ALLN and IL-1β, the ubiquitination of IκBα was clearly increased. Interestingly, curcumin was able to inhibit the IL-1β-induced IκBα ubiquitination, in the presence or absence of inhibitor (Fig. 4c). These results clearly show that curcumin affects the phosphorylation and ubiquitination of IκBα.

3.6. *Curcumin blocks IL-1β-induced Akt Activation*

The activation of IKKα is a prerequisite for the phosphorylation of IκBα. It has been reported that pro-inflammatory cytokine mediated activation of IKKα is associated with an upstream protein kinase, Akt (serine-threonine kinase, protein kinase B), and in turn Akt mediates IKKα phosphorylation [31]. To further test whether curcumin blocks the IL-1β-induced IKBα phosphorylation is due to inhibition of Akt, the serum starved chondrocytes were treated with IL-1β (10 ng/ml) for different indicated times and then co-treated with curcumin (50 nM) for 4 h. As shown in Fig. 5, I, IL-1β-induced activation of Akt in a time-dependent manner. In co-treated chondrocytes, curcumin clearly inhibited the activation of Akt (Fig. 5, I). Taken together, these results indicate that curcumin blocks IKK activation through inhibition of an upstream protein kinase Akt. Synthesis of the Akt protein remained unaffected (Fig. 5, II).

4. Discussion

The results presented here lead to the following findings: (1) curcumin inhibits the IL-1β- and TNF-α-mediated suppression of key extracellular matrix and signalling proteins in human chondrocytes. (2) Curcumin is able to antagonize the IL-1β- and TNF-α-dependent up-regulation of MMP-9 and COX-2. (3) IL-1β results in phosphorylation and nuclear translocation of the p65 NF-κB subunit. In addition, time-dependent phosphorylation, degradation and the ubiquitination of the inhibitory subunit of NF-κB: IκBα is evident in the response to IL-1β treatment. (4) The suppression of NF-κB activation by curcumin is accompanied by the inhibition of phospho p65 translocation to the chondrocyte nucleus. (5) These molecular events may be accounted for by inhibition of IL-1β-induced Akt-phosphorylation, a process we believe to be mediated by curcumin (Fig. 6).

4.1. *Curcumin inhibits IL-1β and TNF-α mediated extracellular matrix and integrin degradation*

IL-1β and TNF-α resulted in down-regulation of expression of collagen type II and cartilage-specific proteoglycan. In our *in vitro* model system of human chondrocytes [9,26] we have consistently observed a time-dependent decrease in the synthesis of cartilage-specific extracellular matrix components such as collagen type II, a finding that is in complete agreement with previous *in vitro* studies [4,7,8,32]. Treatment with curcumin led to a recovery of collagen type II synthesis in IL-1β- and TNF-α-stimulated chondrocytes.

Interactions between the extracellular cartilage matrix and chondrocytes are important for the proliferation, differentiation, and survival of the cells [24,27,33] since inhibition of cell–matrix interactions leads to chondrocyte apoptosis [33].

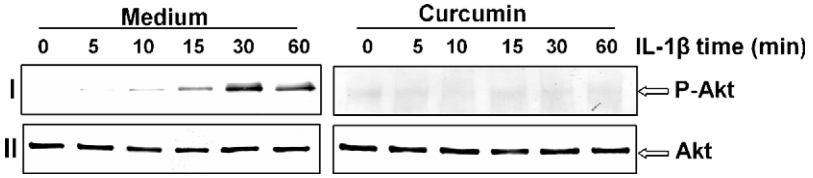

Fig. 5 – Effect of curcumin on IL-1β-induced Akt activation. Serum-starved human articular chondrocytes (0.1×10^6 cells/ml) were treated with 10 ng/ml IL-1β for the indicated times. Identical cultures were co-treated with 50 μM curcumin for 4 h at 37 °C. Cell extracts were investigated for expression of Akt by western blot analysis using anti-Akt and anti-phospho-Akt antibodies. Treatment of chondrocytes for 10 min with 10 ng/ml IL-1β increased the phosphorylated form of Akt in a time-dependent manner (I). Conversely, phosphorylation of Akt was strongly inhibited by curcumin in co-treated cultures. Synthesis of the Akt remained unaffected (II). Data shown are representative of three independent experiments.

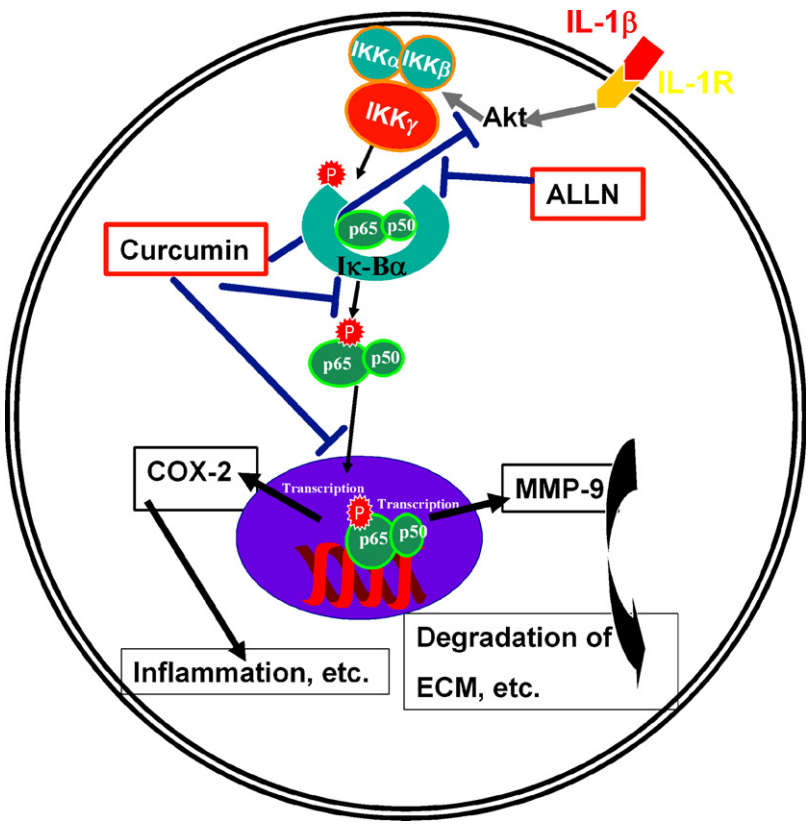

Fig. 6 – Working model of curcumin's anti-inflammatory actions on IL-1β- and TNF-α- induced signal transduction in chondrocytes. Binding of IL-1β to its receptor activates IKKγ kinase through which Akt phosphorylates IκBα the inhibitory unit of the NF-κB (p65/p50) complex in the cytoplasm. IκBα dissociates from the complex releasing NF-κB which is now free to translocate to the nucleus and modulate the transcription of its target genes. NF-κB up-regulates a large number of pro-inflammatory genes including MMP-9 and COX-2. Moreover, NF-κB mediates down-regulation of key matrix and signalling proteins such as collagen type II and β1-integrin in chondrocytes. Curcumin inhibits IκBα-degradation thus preventing NF-κB activation and NF-κB translocation to the nucleus. Its biological actions prevent NF-κB activation and inhibit cytokine-induced activation of pro-inflammatory enzymes in chondrocytes.

Cell–matrix interactions are primarily mediated via multi-functional β1-integrins [33] which organize cell surface mechanoreceptor complexes [34] and function as signal transduction molecules [35] stimulating MAPkinase pathways [24]. In this study down-regulation of β1-integrins in response to IL-1β or TNF-α stimulation was evident. The decreased expression of signalling proteins such as β1-integrins may be a secondary effect of reduced cell–matrix interactions. Our previous studies and those of other investigators support the notion that reduced cell–matrix interactions lead to inhibition of Erk1/2 signalling and stimulate the apoptotic pathway in human chondrocytes [27,34–36]. Curcumin-treatment leads to the recovery of these IL-1β-repressed intracellular signalling proteins. It remains unclear whether NF-κB-inhibition by curcumin is directly responsible for the restored synthesis of β1-integrins or whether the inhibition of IL-1β-mediated matrix degradation by MMPs improves cell–matrix interactions and hence β1-integrin-associated Ras-MAPkinase signalling [24]. The intriguing question that arises is whether certain signalling proteins such as integrins are directly and negatively regulated by NF-κB. Interestingly, some adhesion molecules such as I-CAM/V-CAM have been shown to be regulated by NF-κB [19]. Moreover, curcumin has been shown to suppress cell surface expression of adhesion molecules ICAM-1 and VCAM-1 on monocytes, inhibiting their adhesion and inflammatory effects [37,38].

4.2. *Curcumin antagonizes up-regulation of pro-inflammatory enzymes by IL-1β and TNF-α*

It is generally accepted that MMPs function as matrix degrading enzymes mediating extracellular matrix macromolecule remodelling [39]. In physiological conditions, an equilibrium exists between MMP activity and new matrix synthesis; in this scenario expression of MMPs and tissue inhibitors of MMPs (known as TIMPs) is tightly regulated and balanced against synthesis of new extracellular matrix proteins. However, in arthritis this fine balance between synthesis and degradation goes astray; unregulated MMP expression and activity results in excessive extracellular matrix degradation and leads to cartilage destruction [40]. This study has shown that IL-1β and TNF-α both induce time-dependent up-regulation of MMP-9 as has been previously reported for other MMPs [4,5,41]. COX-2 is an important

mediator of pain and inflammation in osteoarthritic joints [42]. COX-2, but not COX-1 production is stimulated by IL-1β and TNF-α [5,41]. COX-2 activity leads to PGE_2 and thromboxane production [12,43,44]. PGE_2 exerts some additional catabolic effects on chondrocytes such as decrease in proliferation of chondrocytes and inhibition of proteoglycan synthesis [44]. In this study, unstimulated human chondrocytes only showed traces of COX-2 expression. However, in the presence of IL-1β- or TNF-α-stimulation COX-2 levels increased. Here we have shown, for the first time, that curcumin inhibits the synthesis of MMP-9 and COX-2 in IL-1β- and TNF-α-stimulated chondrocytes. We propose that this occurs via NF-κB inhibition, because the expression of this [45] protein is regulated by NF-κB as has been shown by other investigators [45,46].

4.3. *Curcumin blocks IL-1β-induced phosphorylation and nuclear translocation of p65*

Many of the downstream effects of IL-1β and TNF-α stimulation such as MMP-up-regulation, COX-2-expression, and NO-generation are regulated by activation of the ubiquitous transcription factor NF-κB [5,15,16,41,47,48]. In the inactive state, the p65 subunit of NF-κB is retained in the cytoplasm, but when NF-κB is activated, the phosphorylated p65 subunit of NF-κB translocates to the nucleus, where it binds to consensus regulatory DNA sequences directly regulating gene expression or trans-activating other transcription factors that possess NF-κB binding sites (e.g. ESE-1) [49]. In the present study, increased phosphorylation of p65 in response to IL-1β was clearly demonstrated by western blot analysis of nuclear extracts of chondrocytes which is also suggestive of the translocation of phospho p65 to the nucleus since the phosphorylated form of p65 decreased in the cytoplasmic extracts. Translocation of phosphorylated p65 to chondrocyte nuclei could also be demonstrated by immunofluorescence microscopy. The mechanism of NF-κB translocation has not yet been elucidated, but may partly depend on phosphorylation of the p65 subunit. Immunofluorescence microscopy confirmed that curcumin inhibits translocation of phosphorylated p65 to the nucleus. In response to curcumin and IL-1β, levels of phosphorylated p65 decrease in cytoplasmic and nuclear extracts as shown by western blotting suggesting inhibition of NF-κB phosphorylation by curcumin.

4.4. *Curcumin affects the dynamics of IκBα and Akt by IL-1β in chondrocytes*

In the present study, curcumin inhibited both IL-1β-induced NF-κB activation by phosphorylation of p65 and phosphorylation of IκBα which, in turn, lead to its degradation and consequently to the release of activated NF-κB. Phosphorylation of IκBα was shown in cytoplasmic extracts in response to IL-1β after 10 min. Later, phosphorylated IκBα decreased in cytoplasmic extracts probably as a result of IκBα degradation. In response to IL-1β and curcumin, phosphorylation of IκBα was inhibited in cytoplasmic extracts.

It is well known that IκBα-phosphorylation is dependent on IKK and kinases that regulate IKK-activation, like Akt and MEKK1 (mitogen-activated protein kinase/extracellular signal-regulated kinase-1) [29]. Furthermore, the activation of IKK by cytokines accompanied the activation of the upstream kinase Akt [31]. In the present study, we have shown that curcumin inhibits IL-1β-induced Akt phosphorylation. It has been shown that Akt stimulates IKK-α phosphorylation at threonine 23. The phosphorylation of this amino acid results in phosphorylation of IKK by Akt or cytokines and consequently, activation of NF-κB [31]. Indeed, the inhibition of IκBα phosphorylation, degradation, ubiquitination and, subsequent phosphorylation and nuclear translocation of p65 by curcumin in this study can be explained, because of the suppression of Akt phosphorylation by curcumin in chondrocytes.

In recent years, NF-κB has been demonstrated to be a key downstream target of IL-1β- and TNF-α-signalling [5,12]. This knowledge has revealed that central transcription factors activated by pro-inflammatory cytokines are promising and realistic new targets for the treatment of degenerative osteoarticular and rheumatic diseases. Recent work on curcumin in clinical trials suggests that it is non-toxic, clinically and pharmacologically safe and suitable for testing as a potential metabonomic therapeutic compound [38,50]. Indeed, curcumin's bioavailability and cellular metabolism is species-specific and will remain controversial for the time being. However, its pharmacological safety, combined with its potent ability to prevent IL-1β- and TNF-α-induced activation and down-regulation of pro-inflammatory enzymes such as MMP-9 and COX-2, and up-regulation of cartilage-specific matrix components and important signalling proteins in chondrocytes, provide sufficient rationale for its use as a small-molecule chemopreventive agent in further *in vitro* studies and clinical trials in carefully selected human patients and animal models of OA.

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